The results described above clearly demonstrate that the augmentation of contractile force in the presence of pH 7.8 depends mainly upon the enhanced transmembrane Ca influx during excitation. In order to see whether alkalosis exerts its positive inotropic effect on principle via an increase of Ca current, in a second type of experiments pH was elevated by means of a stronger HCO_3 concentration (23.8 m*M*) with simultaneous reduction of the CO_2 concentration to 1%. Figure 3 shows the evaluation of these experiments. Again, within 15 min an increase of isometric contraction force occurred which amounted to 28.7% on an average. A longer time of exposure to the alkalotic Tyrode solution had no additional effect. However, a clearly different response of the Ca action potential was obtained; 10 min after switching to this medium a slight decrease of its upstroke velocity by 14.5% appeared. It was followed by a rise attaining the control values of upstroke velocity 5 min later. Obviously, the increase of contractile force is not accompanied with proportional changes in transmembrane Ca current. The same phenomenon was found under the influence of cardiac glycosides (THYRUM[7]; NAWRATH et al.[8]), which proves that an enlargement of Ca influx during excitation has not to be considered as an essential prerequisite for the positive inotropic action in heart muscle. Rather the underlying increase in activator Ca can be caused predominantly by changes of intracellular Ca movements as well. In the case of extracellular alkalosis (produced by increased HCO_3 and reduced CO_2 concentration) the augmented contractile activity seems to results from promoting the Ca release from stores probably induced by concomitant changes of the intracellular H concentration. If this leads to a rise in intracellular free Ca, a plausible explanation for the unexpected cessation of the augmentation of Ca currrent becomes available.

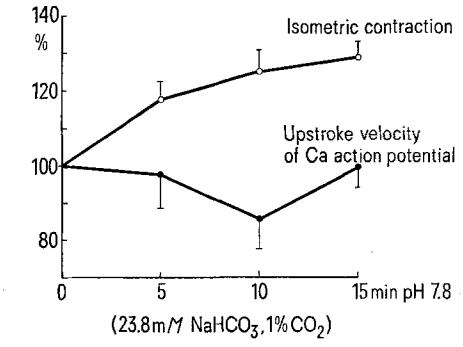

Fig. 3. Changes of upstroke velocity of Ca action potential (filled circels) and isometric contraction (open circles) following perfusion with a 23.8 m*M* HCO_3- and 1% CO_2-containing medium (pH 7.8). Each point represents the mean value from 5 experiments (vertical bars: standard deviation).

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5-Thio-D-Glucose: *o*-Diphenoloxidase Inhibition as its Mechanism of Action

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Summary. 5-Thio-D-glucose completely inhibited *o*-diphenoloxidase from animal as well as plant sources. It has been reported that thioglucose suppresses spermatogenesis in mice and also insect metamorphosis, probably through inhibition of glucose transport. Inhibition of *o*-diphenoloxidase (which is active in spermatozoa and insect larvae) is suggested as an alternative mechanism of action of thioglucose.

This communication deals with a possible mechanism of action of 5-thio-D-glucose, which has been reported to be a unique male contraceptive[1]. In thioglucose, which was synthesized in 1962, the ring oxygen of glucose is replaced by a sulfur atom[2]. Feeding of male mice with the compound resulted in testicular atrophy and total inhibition of spermatogenesis[1]. The effect was fully reversible on withdrawing the drug. When thioglucose was fed to larvae of *Drosophila melanogaster*, metamorphosis of the larvae was suppressed[3]. Although the drug inhibits competitively the active transport of D-glucose across cell membranes[4], the exact mechanism of action of thioglucose on spermatogenesis or insect metamorphosis has not yet been established.

It has been pointed out that, since glucose is a major energy source for brain metabolism, an antagonist of glucose could have serious side effects[5]. However, no acute toxic effects were observed in experimental animals given large doses of the drug, and LD_{50} of thioglucose was quite high (14 g/kg body weight)[4]. Evidently, the compound might have some other mode of action as well, besides inhibiting glucose transport. Spermatozoa contain an enzyme which actively oxidizes dopa to pigment[7–9], and sulfur-containing compounds are known to be inhibitors of *o*-diphenoloxidase (tyrosinase) (*o*-diphenol: oxygen oxidoreductase)[10]. Moreover, tryosinase is known to have an important role in insect development[11].

Methods. To study the effect of 5-thio-D-glucose on *o*-diphenoloxidase, the oxidation of dopa (3,4-dihydroxyphenylalanine) by cultured melanoma cells and by lyophilized mushroom tyrosinase was tested with and without the drug. Mushroom tyrosinase and 5-thio-glucose

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Table I. Effect of thio-D-glucose on *o*-diphenoloxidase

Reaction system	Absorbance 475 nm		Oxygen uptake: nmoles	
	Melanoma cells	Mushroom tyrosinase	Melanoma cells	Mushroom tyrosinase
Enzyme + Dopa	0.100	0.110	255.95	289.33
Enzyme + Dopa + thioglucose	0	0	0	0
Enzyme + Dopa + glucose	0.100	0.110	—[a]	—

[a] Not done.

were obtained from the Sigma Chemical Company, St. Louis, Missouri, USA. Melanoma cells were purchased from the American Type Culture Collection (CCL 53.1), and grown in Ham's F-10 medium at 37 °C. L-Dopa was obtained from the ICN Life Sciences Group, Cleveland, Ohio, USA. The reaction system and the experimental conditions were as follows: Na_2HPO_4-KH_2PO_4 buffer (pH 6.8), 0.1 *M*; L-dopa, 0.002 *M*; 5-thio-D-glucose or D-glucose, 0.004 *M*; melanoma cells, 1.8 mg protein; mushroom tyrosinase, 10 µg; temperature, 37 °C; time, 30 min; volume, 3 ml. After incubation, the spectrum of the quinone (dopachrome) formed from dopa was determined spectrophotometrically at wavelength 475 nm. The readings were corrected for any absorbance due to enzyme, substrate or inhibitor. Oxygen uptake was measured polarographically using a Gilson Oxygraph equipped with a Clark oxygen electrode. The results were corrected for endogenous respiration. The reaction system was similar to the above, except that the volume was 2 ml and the buffer concentration 0.05 *M*.

Results. The results given in Table I show that thioglucose completely inhibits *o*-diphenoloxidase from mammalian and plant sources; D-glucose, by itself has no effect. Concentrations as low as 2.5×10^{-4} *M* of the compound produced total inhibition of *o*-diphenoloxidase; at 1.25×10^{-4} *M* level, the inhibition was 78%. To ascertain whether the inhibitory effect would be overcome with time, the reaction mixtures were left overnight at room temperature (23–25 °C). The samples without inhibitor underwent further oxidation and polymerization to melanin pigment. However, even after 24 h no oxidation was evident in the samples containing the compound (Table II). The lowest concentration of the drug which prevented melanin formation was 5×10^{-4} *M*; below this level, the effect was only partial.

Discussion. The results show that thio-D-glucose is a potent inhibitor of *o*-diphenoloxidase. It is known that spermatozoa contain an enzyme which oxidizes dopa to melanin pigment[7–9]. Although no direct evidence is available to support the hypothesis, it is likely that tyrosinase has a significant metabolic role in sperm development and thioglucose suppresses this enzyme activity of the reproductive cells. Tyrosinase is also active in insect metamorphosis. The enzyme is responsible for the sclerotization of insect cuticle[11]; phenoloxidase is functional at all stages of insect development, and a strong stimulation of the enzyme occurs just before larval-pupal ecdysis[12]. The failure of *Drosophila* larvae to undergo metamorphosis when fed thioglucose might be due to inhibition of tyrosinase by the drug. In our studies, insect tyrosinase was found to be a particulate enzyme like tyrosinase occurring in melanocytes[13]; the *o*-diphenoloxidase of spermatozoa might also be similar. Presumably, the suppression of phenoloxidase activity is a more potent effect of thio-D-glucose than inhibition of glucose transport; the compound produces infertility in mice at concentrations far below those needed for causing hyperglycemia[1]. A structural analog of dopa, mimosine was reported to cause reversible infertility in female rats[14]. We have shown that mimosine produces total inhibition of mammalian and plant tyrosinases[15]. These studies also indicate that *o*-diphenoloxidase may have a key role in the normal functioning of reproductive cells.

Further studies dealing with inhibition kinetics, and with the direct effect of thioglucose on tyrosinase of reproductive cells and of insect larvae will be reported later. Experiments are also in progress to assess the influence of the drug on both oogenesis and spermatogenesis in mice. Preliminary results with *o*-diphenoloxidase of *Tenebrio molitor* larvae show that thioglucose inhibits insect tyrosinase as well; higher concentrations of dopa reverse the inhibition, suggesting that thioglucose acts as a competitive inhibitor of tyrosinase.

Table II. Effect of thio-D-glucose on melanin formation

Reaction system	Absorbance 400 nm (for melanin)	
	Melanoma cells	Mushroom tyrosinase
Enzyme + Dopa	0.700	0.780
Enzyme + Dopa + thioglucose	—	—
Enzyme + Dopa + glucose	0.730	0.800

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